

STUDIES ON OÖGENESIS IN THE POLYCHAETE ANNELID *NEREIS GRUBEI* KINBERG. I. SOME ASPECTS OF RNA SYNTHESIS

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Oögenesis in the marine polychaete *Nereis* appears to include at least two distinct growth phases. Oöcyte growth is slow for much of the period available for oögenesis, and more rapid during the period of somatic metamorphosis which precedes spawning (Clark and Ruston, 1963). Both somatic metamorphosis and rapid oöcyte growth can be initiated by decapitation, which is thought to deprive the animal of a prostomial source of an inhibitory hormone (Durchon, 1952; Hauen-schild, 1956). Inasmuch as the suggestion has been made that an alteration in RNA metabolism is associated with the increased growth rate of the oöcytes (Durchon and Boilly, 1964), we have examined the types of RNA normally formed during the rapid growth period, which in *Nereis grubei* commences when the oöcytes are 90-100 μ in diameter. Oöcyte morphology changes considerably through this period (Spek, 1930); in *N. grubei* the oöcyte is blue-green and opaque for most of the period of rapid growth. When it reaches 170-180 μ in diameter, the color changes to a brilliant yellow-green. Still later, the lipid yolk coalesces into large translucent droplets around the nucleus, while granules of jelly precursor material condense at the cortex of the oöcyte, which is about 200 μ in diameter at maturity. Mature oöcytes thus contain a yellow-green and strikingly layered cytoplasm, and are distinct in appearance from the opaque, homogeneous, blue-green 160 μ oöcytes. RNA synthesis during the rapid growth period has been examined in cells in each of these morphological stages.

MATERIALS AND METHODS

Animals

Female specimens of *N. grubei* of appropriate age were collected at several points in central California (Moss Beach, San Mateo County; Pacific Grove, Monterey County). They were maintained in the laboratory at 11-15° C in sea water containing 100 μ g/ml streptomycin base and 100 units/ml penicillin.

Incubation of oöcytes with tritiated uridine

Animals anesthetized in 7.3% MgCl₂ were passed through a series of five changes of sterile sea water, with vigorous agitation between changes. The animal

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was then split open; the oöcytes were removed in as sterile a manner as possible, placed upon sterile Nitex nylon bolting cloth ($44\ \mu$ or $64\ \mu$ mesh) and rinsed repeatedly with sterile sea water until phase microscopic examination indicated the absence of all coelomic cells. The oöcytes were then placed in 5 ml sterile sea water containing $35\ \mu\text{C}$ of H^3 -uridine (New England Nuclear. Specific activities are given in the figure legends).

Those species of *Nereis* which breed in the heteronereid state appear to swarm soon after the oöcytes have reached the mature condition. Females deprived of males during this period will swim actively for a period of hours, then sink to the bottom. Soon thereafter, the oöcytes and the whole coelomic mass become gelatinous and the oöcytes begin to degenerate. The "fresh mature" oöcytes were taken from an animal actively swimming at the time of oöcyte removal. The "old mature" oöcytes were from an animal which had been swimming the previous day. These cells maintained excellent morphological integrity, but could be expected to disintegrate within 24–48 hours.

Upon completion of the incubation period, an aliquot of cells was withdrawn and fixed for one hour in acetic acid-ethanol (1:3) for autoradiography. The balance was frozen in a dry-ice-acetone bath for subsequent RNA extraction.

Autoradiography

After fixation the oöcytes were stained lightly with eosin to enhance visibility, imbedded in paraffin and sectioned at $5\ \mu$. After paraffin removal, the sections were dipped in ice-cold 5% trichloroacetic acid for 15 minutes (not longer), rinsed, and coated with Ilford K5 liquid nuclear emulsion. Exposure was from 10 days to 3 weeks. They were then developed for 6 minutes in Kodak D19 (18°C) and stained in Mayer's hemalum and celestine blue (Doniach and Pelc, 1950). Successful RNase controls required the use of 1 mg/ml RNase (Worthington) for 17 hours (*i.e.* overnight) at 37°C . [Tweedell (1966) also found somewhat extreme conditions to be necessary for this reaction in *Pectinaria* oöcytes.]

RNA extraction

RNA was extracted by the cold-phenol method of Brown and Littna (1964), including DNase digestion (Worthington, 1x crystallized). The concentration of sodium dodecyl sulfate for the initial phenol extraction was increased to 2%. With two exceptions (the preparations in Figures 8b and 8c), the samples were also digested with 50–100 μg Pronase (Calbiochem; previously autodigested) for an additional 30 minutes at 18 – 20°C . The samples were then brought to 0.5% SDS and 0.1 *M* NaCl and re-extracted with an equal volume of ice-cold phenol. The aqueous layer was re-extracted with chloroform at least twice more, and the RNA again precipitated with the addition of two volumes of absolute ethanol.

Analysis of extracted RNA

For sucrose gradient analysis, aliquots (1 ml or less) of the RNA solutions in 0.01 *M* sodium acetate, pH 5 were layered on top of 25 ml linear 5–20% sucrose gradients, made up in 0.01 *M* sodium acetate, 0.10 *M* NaCl and 10^{-4} *M* EDTA.

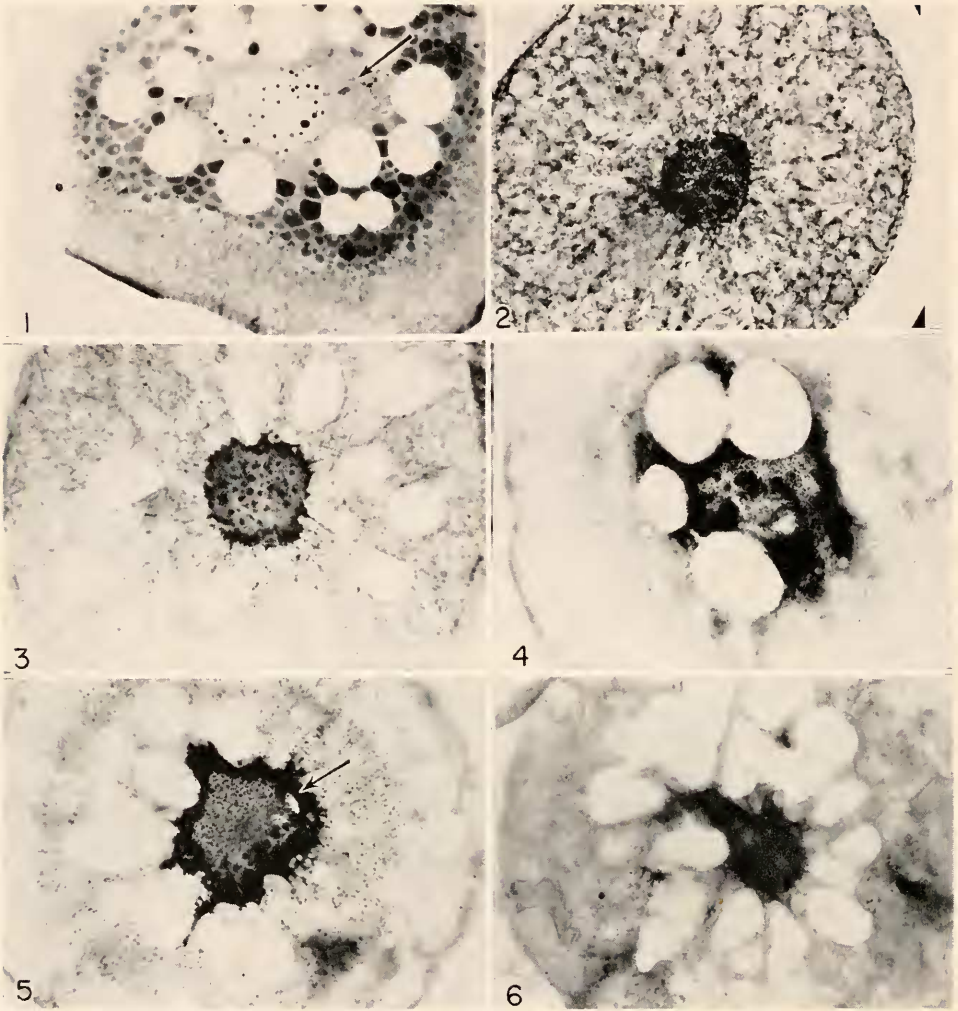


FIGURE 1. One-micron section of a mature oocyte from *Nereis grubei* imbedded in maraglas and stained with azure II-methylene blue. Note the reticulated area of cytoplasm surrounding the nucleus but clearly distinct from it (arrow).

FIGURE 2. Autoradiogram of 165 μ oocyte (G485), blue-green in life, showing nuclear localization of silver grains.

FIGURE 3. Autoradiogram of 190 μ oocyte (G257), yellow-green in life and showing concentration of lipid yolk but incomplete cytoplasmic stratification. Nuclear localization of silver grains, with local concentrations above nucleoli.

FIGURE 4. Autoradiogram of mature oocyte (G359) incubated 4 hours with H^3 -uridine. Nuclear concentration of label, with relatively little over basophilic perinuclear cytoplasm.

FIGURE 5. Autoradiogram of older mature oocyte (G548) with both nuclear and cytoplasmic label; the cytoplasmic label is concentrated over the basophilic perinuclear cytoplasm.

FIGURE 6. Autoradiogram of oocyte from same sample as Figure 5, treated 17 hours with RNAase. Slight residual nuclear label, disappearance of nucleoli, perinuclear cytoplasmic basophilia and most silver grains.

Aliquots for RNase digestion were adjusted to pH 8 with 0.1 M Tris-HCl and incubated 10 minutes or longer at 18–20° C with 10–20 μ g of boiled RNase (Worthington). The extracted RNA showed none of the resistance to the enzyme shown by that in the autoradiograms. The gradients were centrifuged 9–10 hours at 2° C and 25,000 RPM in the SB-110 rotor of an International B-60 ultracentrifuge. Fractions were diluted to 3 ml with 0.01 M sodium acetate, pH 5, for determination of the optical density at 260 m μ in a Beckman DU spectrophotometer, precipitated in the cold with 0.1 mg bovine albumen carrier and 0.3 ml 50% trichloroacetic acid, collected on glass fiber filters (Reeve-Angel) and counted in a Nuclear-Chicago liquid scintillation spectrometer with 4 ml toluene scintillator (4 g PPO and 0.2 g POPOP per liter of toluene).

DNA base composition

Two gamete-bearing male *Nereis* were homogenized in 5 ml SET (0.1 M NaCl, 0.1 M EDTA, 0.01 M Tris-HCl, pH 8) and warmed immediately to 60° C. Twelve mg Pronase (Calbiochem; previously autodigested) and sodium dodecyl sulfate to 0.5% were added, and incubated for 2½ hours. The solution was then cooled to room temperature and extracted by gentle rocking with an equal volume of water-saturated phenol. Following centrifugation at 12,000 g the aqueous layer was removed, re-extracted with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged as before. Two volumes of absolute ethanol were added to the aqueous extract; the precipitate was collected and redissolved in 4 ml SET diluted 1:1 with water. The solution was then incubated with 200 μ g RNase (Worthington; previously boiled 10 minutes) for 2½ hours at 37° C. Following addition of 500 μ g Pronase, the incubation was continued for an additional hour. The DNA was then precipitated with ethanol, redissolved in SET, and extracted twice with phenol and once with chloroform-isoamyl alcohol as before. The resulting ethanol precipitate was redissolved in SET for CsCl density gradient analysis. We are indebted to Dr. Philip Hanawalt for carrying out this analysis. The guanine-cytidine content of the DNA was calculated from its buoyant density in CsCl by the method of Schildkraut, Marmur and Doty (1962).

RESULTS

Buoyant density of Nereis DNA

The buoyant density in CsCl of a sample of *Nereis* DNA extracted as described above was found to be about 1.700 g cc⁻³, which corresponds to a guanine-cytidine content of 40–41%. Although this information could not be put to the use originally intended in this analysis, we feel that it may be helpful to record this figure for the benefit of future investigations of nucleic acid metabolism in *Nereis*.

Autoradiography

The autoradiograms indicate the presence of labeled uridine in the nucleoli of cells in each size class. Label is also present over non-nucleolar regions of the germinal vesicle in all samples (Figs. 2–5). However, prominent cytoplasmic label

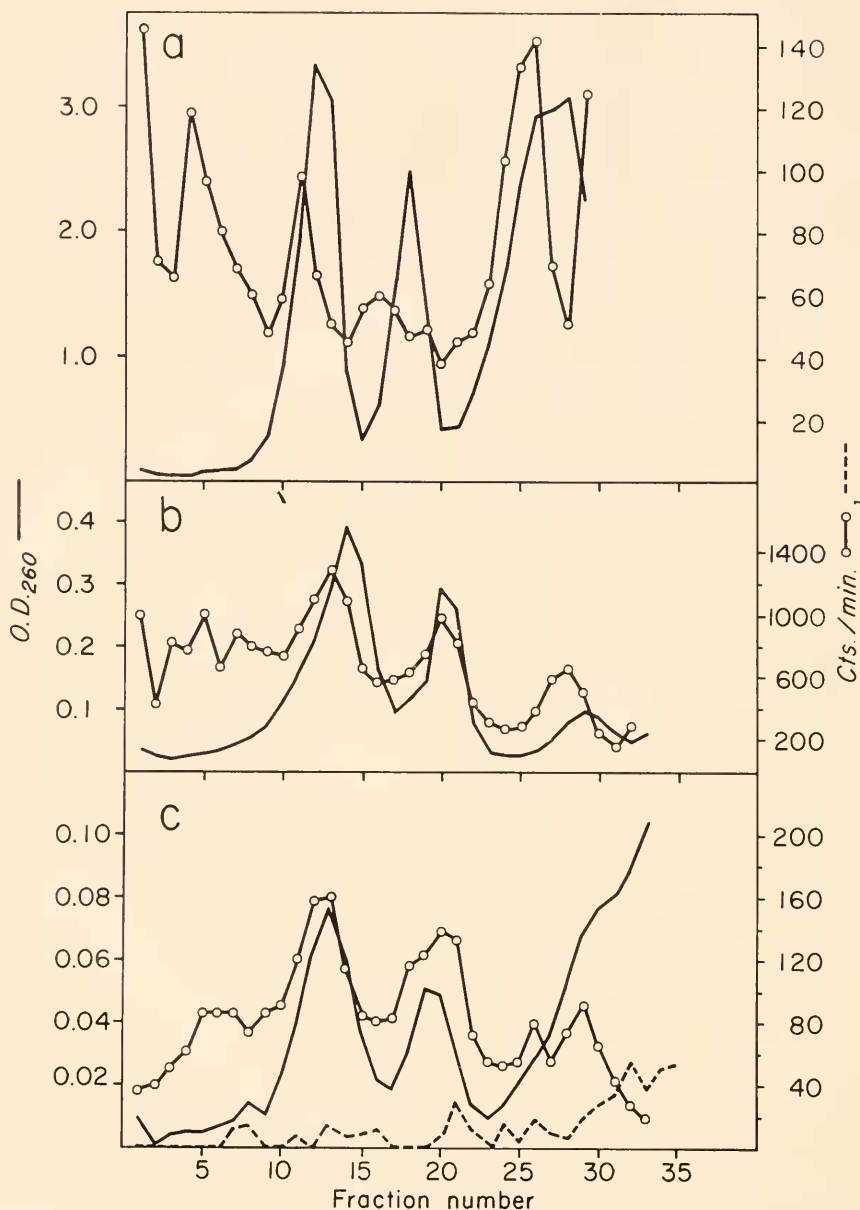


FIGURE 7. Sucrose gradient analysis of RNA from oocytes of immature *Nereis grubei*. Open circles: radioactivity; solid line: optical density at 260 mμ; broken line: radioactivity after treatment with RNase. Specific activity of H^3 -uridine applied in each case is given in parentheses following the incubation time. (a) 100 μ oocytes, 4 hours incubation (24.5 C/mM). *Urechis caupo* carrier RNA added. (b) 165 μ oocytes, 4 hours incubation (3.6 C/mM). No carrier RNA added. (c) 190 μ oocytes, 4 hours incubation (24.5 C/mM). No carrier RNA added.

was found only in the two samples of mature oöcytes incubated for 21 hours with the precursor.

One-micron sections of mature oöcytes imbedded in Maraglas (Fig. 1, arrow) reveal that the basophilic region seen at the nuclear margin in paraffin sections (*e.g.* Fig. 5, arrow) is actually cytoplasmic. This basophilia is present to only a limited extent in 190 μ oöcytes (Fig. 3) and appears to represent a facet of the general layering of cytoplasmic elements which occurs during the final stage of oöcyte development. Cytoplasmic labeling in mature oöcytes incubated for 21 hours with H^3 -uridine is concentrated in this region of the cytoplasm (Fig. 5) although the region is only slightly labeled in mature oöcytes incubated for four hours (Fig. 4). Both the incorporated radioactivity and the basophilia of this region are removed by treatment with RNase (Fig. 6). A preliminary examination of this region with the electron microscope indicates that it does not consist of the stacked annulate lamellae (also basophilic) found in artificially accelerated oöcytes of *Nereis diversicolor* by Durchon and Boilly (1964); (Durchon, 1967, p. 130 and our own observation).

Sedimentation analysis of extracted RNA

The sedimentation patterns of RNA extracted from *Nereis* oöcytes are presented in Figures 7 and 8. The RNA synthesized by immature and mature oöcytes during a 4-hour incubation with H^3 -uridine is distinctly heterogeneous (Figs. 7a and 8a). However, prominent peaks of radioactivity are associated with the 28s and 18s ribosomal RNAs (sedimentation values have not been determined for *Nereis* RNAs, but are given with reference to RNA from other eukaryotic cells) seen by optical density in the preparations from 165 μ and 190 μ oöcytes (Figs. 7b and 7c) and from mature oöcytes labeled for 21 hours (Figs. 8b and 8c). Furthermore the radioactivity profile from the 100 μ oöcytes is consistent with the presence of 45s and 30s ribosomal RNA precursors. Taken together, the sucrose gradient profiles and autoradiograms suggest that ribosomal RNA synthesis occurs at all stages examined.

The low molecular weight RNA synthesized by these oöcytes is not adequately characterized by sucrose gradients; therefore, positive statements about 5s ribosomal (Brown and Littna, 1966) and 4s transfer RNA synthesis are precluded. However, it does appear that mature oöcytes accumulate relatively little radioactivity into low molecular weight RNA when compared with younger oöcytes. This observation could reflect a drastic reduction or cessation of transfer RNA synthesis towards the end of oögenesis.

No differences were found which could be clearly associated with the progressively altered morphology of the oöcytes.

In evaluating these results, the fact that the oöcytes were incubated in sea water rather than coelomic fluid should be kept in mind. We have observed that eleocytes adhering to growing coelomic oöcytes readily separate from them upon transfer to sea water, indicating some change at the oöcyte surface. Furthermore, Gonse (1957a, b) has reported differences in respiratory levels in oöcytes from the sipunculid *Phascolosoma vulgare* which he attributes to an effect of sea water upon cell permeability to sugars.

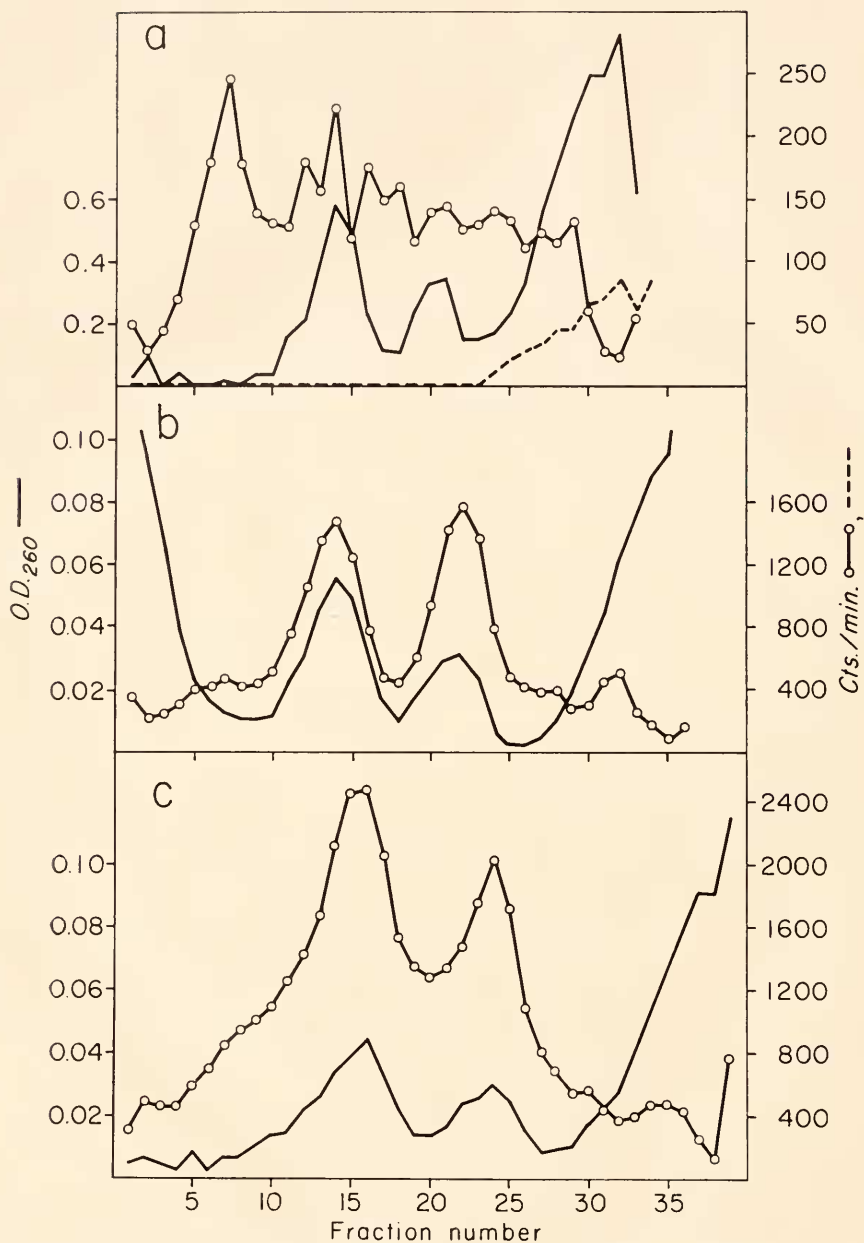


FIGURE 8. Sucrose gradient analysis of RNA from mature oocytes. Legend as for Fig. 7. (a) Mature oocytes 4 hours incubation (24.5 C/mM). No carrier RNA added. (b) "Fresh" mature oocytes, 21 hours incubation (8 C/mM). No carrier RNA added. (c) "Older" mature oocytes, 21 hours incubation (8 C/mM). No carrier RNA added.

DISCUSSION

These studies clearly demonstrate that mature *Nereis* oöcytes, as well as immature oöcytes at the stages examined, are able to synthesize RNA in sea water in the absence of any accessory cells. An active synthesis of RNA has also been observed in the immature oöcytes from a variety of other marine invertebrates. As demonstrated autoradiographically these include sea urchins (Ficq, 1964; Piatigorsky and Tyler, 1967), starfish (Ficq, 1955b), amphibians (Ficq, 1955a), the polychaete worms *Pectinaria* (Tweedell, 1966), *Autolytus* (Allen, 1967) and *Ophryotrocha* (Ruthmann, 1964), and the echiuroid worm *Urechis* (Gould, unpublished data). Sucrose gradient analysis of the RNA synthesized by immature sea urchin (Gross, Malkin and Hubbard, 1965; Piatigorsky and Tyler, 1967) and *Urechis* (Gould, unpublished data) oöcytes has indicated considerable ribosomal RNA synthesis.

Reports of RNA synthesis by mature oöcytes, however, are fewer. Ribosomal RNA, transfer RNA, and RNA with a more DNA-like base composition are synthesized by mature *Urechis* oöcytes (Gould, unpublished data). Small amounts of heterogeneously sedimenting radioactive RNA have been detected in mature sea urchin oöcytes (Siekevitz, Maggio and Catalano, 1966), but studies with mature sea urchin oöcytes have generally been frustrated by the poor penetration of RNA precursors into these eggs (Piatigorsky, Ozaki and Tyler, 1967). The finding that mature *Nereis* oöcytes incorporate considerable amounts of precursor into RNA further discredits the idea that all mature oöcytes are in a state of metabolic inhibition (see, for example, Monroy, 1965, p. 77).

It should be noted, however, that our results contrast somewhat with the autoradiographic observations of Dhainaut (1965) on the oöcytes of the non-metamorphosing *Nereis diversicolor*. Mature oöcytes (200 μ) of this species were found to contain little cytoplasmic label and relatively little nuclear label after 12 hours in the presence of injected H³-uridine. This author concludes that RNA synthesis virtually ceases in the mature oöcytes of this species, although we have found notable incorporation in three runs with mature oöcytes from *N. grubei*, which metamorphoses to spawn. It would be interesting to know whether this difference is related to the different modes of reproduction in the two species, or to differences of technique.

Previous investigators have found it difficult to study RNA metabolism in immature oöcytes of a given stage in marine invertebrates. Thus Piatigorsky *et al.* (1967) reported results obtained with mixtures of mature oöcytes and immature oöcytes of variable age, obtained from the sea urchin *Lytechinus pictus*. These authors have also examined the RNA accumulated in mature oöcytes gathered from animals injected with the precursor weeks or months previously (Piatigorsky and Tyler, 1967). However, the study of synthetic processes during echinoid oögenesis is hampered by the nature of the sea urchin ovary, in which oöcytes develop asynchronously while imbedded in a matrix of several other cell types (Holland and Giese, 1965). Oöcytes in a given stage of development cannot be conveniently isolated for chemical analysis. The ease with which relatively homogeneous populations of oöcytes in a given stage of oögenesis may be obtained makes *Nereis* an excellent organism in which to investigate the stage specificity of the synthesis of

ribosomes and other materials (*e.g.* yolk components) which accumulate during oögenesis. The fact that the growth rate of these cells is influenced by an inhibitory hormone also makes them attractive biological objects as a hormone target tissue consisting of a single, isolable cell type.

This research was supported by NSF Postdoctoral Fellowship 46015 (PCS), NIH Predoctoral Fellowship 5-F1-GM-21,696 (MCG), NIH Grant GM-10,060 (to N. K. Wessels) and NSF Grant GB-6424X (to H. A. Bern).

SUMMARY

1. RNA synthesis in developing oöcytes of *Nereis grubei* has been studied by autoradiography and by sucrose gradient centrifugation of RNA extracted from oöcytes incubated with tritiated uridine.

2. Oöcyte samples with an average diameter of 100 μ , 165 μ and 190 μ , as well as mature oöcytes (200 μ), all synthesize ribosomal RNA. Labeled uridine was incorporated in the nucleoli of oöcytes from each size class.

3. Oöcytes of all the above stages synthesized heterogeneously sedimenting RNA; mature oöcytes accumulated relatively little label into low molecular weight RNA.

4. No differences in synthetic pattern were found which could be associated with the progressively altered morphology of the oöcytes, but a ribonuclease-sensitive basophilic region, which surrounds the nucleus of mature oöcytes, was found to accumulate label.

5. The cesium chloride buoyant density of DNA isolated from *N. grubei* sperm was found to be about 1.700 g/cc⁻³, which corresponds to a guanine-cytidine content of 40–41%.

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